

# Plasmonic fusion between fibroblasts and skeletal muscle cells for skeletal muscle regeneration

LIMOR MINAI\* AND DVIR YELIN

*Faculty of Biomedical Engineering, Technion, Israel Institute of Technology, Haifa, 3200003, Israel*

\*[minai@bm.technion.ac.il](mailto:minai@bm.technion.ac.il)

**Abstract:** Normal regeneration of skeletal muscle takes place by the differentiation of muscle-specific stem cells into myoblasts that fuse with existing myofibers for muscle repair. This natural repair mechanism could be ineffective in some cases, for example in patients with genetic muscular dystrophies or massive musculoskeletal injuries that lead to volumetric muscle loss. In this study we utilize the effect of plasmonic cell fusion, i.e. the fusion between cells conjugated by gold nanospheres and irradiated by resonant femtosecond laser pulses, for generating human heterokaryon cells of myoblastic and fibroblastic origin, which further develop into viable striated myotubes. The heterokaryon cells were found to express the myogenic transcription factors MyoD and Myogenin, as well as the Desmin protein that is essential in the formation of sarcomeres, and could be utilized in various therapeutic approaches that involve transplantation of cells or engineered tissue into the damaged muscle.

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## 1. Introduction

Natural regeneration of the skeletal muscle involves complex cellular mechanisms in which dormant, muscle-specific stem cells (i.e. satellite cells) either differentiate into myoblasts that fuse together to form new myofibers, or fuse with existing myofibers [1,2] to generate multi-nucleated syncytia. While these processes occur naturally for repairing various muscle injuries, in many cases the muscle damage cannot be naturally repaired, resulting in fibrotic scar tissue and muscle loss of function [3,4]. In various forms of muscular dystrophy, for example, the normal regeneration and activity of the skeletal muscle are severely damaged [5], resulting in progressive weakening and degeneration of the muscle. More than 30 types of muscular dystrophies have been described, where the most common Duchenne muscular dystrophy (DMD) touches 7.1 per 100,000 male children globally [6]. Other common muscular dystrophy diseases include the Becker muscular dystrophy in children [7], and the myotonic and facioscapulohumeral dystrophies in adults [8]. Musculoskeletal injuries that may lead to volumetric muscle loss (VML) are the most prominent injuries in armed combat [9], and sport muscle injuries account for 35–55% of all injuries [10,11].

While most muscle injuries are often treated by rest, physical therapy [12] and surgery [13,14], many types of muscle diseases (i.e. genetic muscle dystrophies) are often treated only by life supporting measures using steroids, ventilatory support and cardiac assistance [15]. The effectiveness of gene therapy by means of gene editing is currently being studied for various types of myopathy [16,17], and cell therapy using satellite cells or stem cell engraftments [18–20] is now being tested in an effort to increase the number of progenitor cells that can differentiate into myoblasts. A main challenge in the transplantation of satellite cells or myoblasts, however, is the gradual loss of their myogenic properties in culture (i.e. *in vitro*), limiting their self-renewal and differentiation capacity, and reducing their ability to regenerate the muscle [21]. Furthermore, extended *in vitro* culturing prior to engraftment often lowers the rate of engraftment success as the cells tend to lose their stem potential [22,23], and while fresh satellite cells could improve

the final outcome, their limited availability makes them less suitable as the sole source of repair. Other techniques involving cell therapy, cell transplantation and tissue engineering are also being studied extensively for treating VML with promising *ex vivo* results [19,23,24].

Effective muscle regeneration could also be triggered by reprogramming healthy (non-muscle) cells into viable muscle cells for strengthening and supporting the damaged muscle tissue. Several research groups have been using viral vectors to insert the MyoD gene into murine-dermal fibroblast cells [25–28], converting dermal fibroblasts into myoblasts and demonstrating the potential of dermal fibroblasts for repairing skeletal muscle tissue. In parallel, Mitani *et al.* have employed Myomaker expression [29] in a variety of non-muscle cells to facilitate their fusion with myoblasts, resulting in nuclear reprogramming for the expression of dystrophin in a culture of *mdx* mice cells, the equivalent of Duchenne muscular dystrophy in human [30]. In both cases, as well as in other studies that involved fibroblast-myoblast cell fusion mediated by Myomaker [31], the expression of MyoD and Myomaker in dermal fibroblasts requires viral vectors for gene transduction, a procedure which may lead to undesired side effects such as mutagenesis and deleterious immune response [16]. Cell reprogramming has also been achieved by using artificial (by adding PEG to the culture medium) fusion between C2C12 mouse muscle cells and eight different human non-muscle cell types including keratinocytes, chondrocytes and fibroblasts, generating heterokaryons that express muscle related genes [32].

Recently, a technique for specific cell-cell fusion has been proposed and demonstrated using femtosecond pulse irradiation of cells whose plasma membrane was conjugated by functionalized gold nanoparticles [33–35]. The technique takes advantage of the unique mechanical and photoionization processes generated around each particle, which destabilize the cells' plasma membrane at multiple locations and lead to effective fusion between neighboring cells. Unlike most approaches for the induction of cell fusion, including incubation with PEG molecules [36], viral transduction of specific cells [37], local electroporation [38] and tightly focused laser beams [39], the high affinity of the gold nanoparticles to the target cells and the local plasmonic interactions allow plasmonic fusion to target only selected cell types within a diverse cell population [34], achieving relatively high fusion efficiencies of only selected cells within heterogeneous cell cultures.

In this study we demonstrate the potential of plasmonic cell fusion for therapeutic applications that involve reprogramming human dermal fibroblasts into viable myoblasts. Using specific attachment of the gold nanospheres to the fibroblast plasma membranes and irradiating the fibroblast-myoblast co-culture by a series of resonant femtosecond pulses, we trigger cell-cell fusion that results in the formation of heterokaryon cells containing nuclei of both myoblastic and fibroblastic origin, which further develop into striated myotubes. The heterokaryon cells remain viable for at least nine days and are shown to express the myogenic transcription factors MyoD [40] and Myogenin [41], indicating differentiation into muscle lineage. The generated myotubes were also found to express the Desmin protein that is known to play an important role in the early formation of myotubes [42]. The results suggest that plasmonic cell fusion could be used to form viable, well-developed myotubes from myogenic and fibroblastic lineage, presenting a potential approach for regenerating damaged muscle tissue.

## 2. Methods

**Cell culture:** Human neonatal dermal fibroblasts (HNDf) with GFP labelled cytoplasm (ANGIO-PROTEOMIE) were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, and 5 mM sodium pyruvate. Human skeletal muscle myoblasts (HSMM, Lonza) were grown in SkGM<sup>TM</sup>-2 skeletal muscle cell growth medium-2 BulletKit (Lonza). Cells were grown under controlled temperature (37°C) and CO<sub>2</sub> concentration (5%). Approximately  $2 \times 10^4$  cells of each type were cultured in each 1 cm<sup>2</sup> well.

*Cell targeting by nanoparticles:* Citrate-capped gold nanoparticles were prepared and coated by PEG and Anti-FIB (Novus Biologicals) according to Weiss *et al.* [43]. Particle diameter was adjusted to 20 nm using sodium citrate reduction. HNDF cells and/or HSMM were grown in eight-well dishes (Thermo Scientific) between 24 to 72 hours, followed by incubation for 18 hours at 37°C with  $2 \times 10^{10}$  nanoparticles. The unbound particles were cleared from the culture medium prior to laser irradiation. Approximately  $10^5$  nanoparticles were bound to each HNDF cell. Control experiments were conducted in parallel to all experimental procedures, including particle-targeted cells without irradiation and irradiation of cells without nanoparticle.

*Laser irradiation:* A beam from an amplified ti:sapphire oscillator (Spitfire Pro XP and MaiTai, Newport Corp.) was tuned to 550 nm wavelength using an optical parametric amplifier (OPA; Topas-C) and was coupled into one of the ports of an inverted microscope for irradiating the cells. Pulse duration was 45 fs at a repetition rate of 1 kHz. For irradiation the cells were placed inside a microscope stage incubator (Okolab Inc.) at controlled temperature and CO<sub>2</sub> concentration. A circular 200- $\mu$ m-diameter Gaussian beam was used to irradiate a total area of 1 cm<sup>2</sup> using a matrix of 50  $\times$  50 spots, where the lateral beam scan rate was adjusted to obtain the desired number of pulses illuminating each sample location. Cells were irradiated by 20 pulses at  $7 \times 10^{10}$  W/cm<sup>2</sup> peak irradiance. Each irradiation experiment was repeated at least three times.

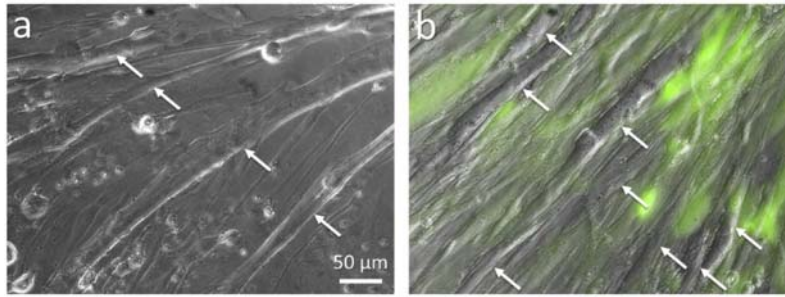
*Cell differentiation into myotubes:* Matrigel was used to support myotube growth [44], as myoblasts grow faster with an ECM component and show higher levels of myogenic differentiation markers [45]. Five hours after irradiation the cells in each well were washed in PBS and covered by Matrigel (300  $\mu$ l, 3 mg/ml total protein). To induce differentiation into myotubes, the cells were supplemented with starvation medium composed of DMEM-F12 (Biological Industries) and 5% serum-free B-27<sup>TM</sup> (Fisher Scientific) for 3–4 days. Myotubes were counted manually based on cell morphology, cell volume, visible striations, total cell length and the number of nuclei (3 or more) [46].

*Immunolabeling:* 8–10 days after irradiation the cells were fixed using 4% paraformaldehyde solution (Santa Cruz) for 20' at room temperature, followed by 3 PBS washes. Cells were then perforated using 0.075% Tween for 3' followed by two 0.05% Tween incubations for 30', and blocked with 5% BSA (Sigma) for 2 hours. Cell perforation of the HNDF cells induced leakage of the GFP cytoplasmic protein, generally resulting in a less uniform, weaker fluorescence labelling. The cells were incubated with the following antibodies: anti MyoD1 (EP212, Cell Marque), Anti Myogenin (EP162, Cell Marque) and anti Desmin (EP15, Cell Marque). Alexa-fluor 647 and 477 Donkey anti-Rabbit or Donkey anti mouse (Jackson ImmunoResearch laboratories inc.) were used as the secondary antibodies. Desmin labelling did not always label the entire myotube and often resulted in patched labelling, probably depending on myotube perforation efficiency and sarcolemma thickness.

### 3. Results

The effective growth of human skeletal muscle myoblasts (HSMM) and their differentiation into viable myotubes following serum depletion was first established by adding Matrigel to the monoculture for supporting the resulting multinucleated myotubes [47] (Fig. 1(a), myotubes marked by arrows). Co-culturing the HSMM cells with supporting GFP-labelled human neonatal dermal fibroblasts (HNDF) resulted in myotubes which were somewhat shorter and thinner but highly aligned and directional (Fig. 1(b), white arrows).

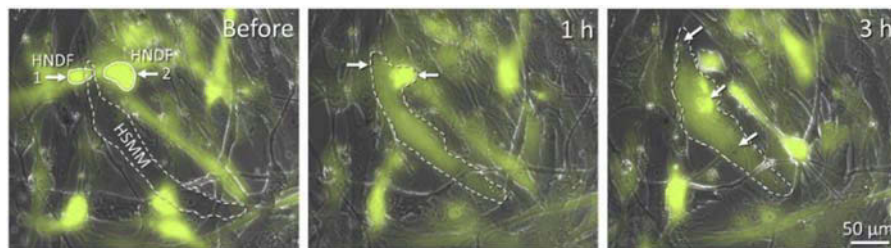
Plasmonic fusion between the HSMM and the HNDF cells was achieved by first incubating the HNDF-HSMM co-culture (approximately 1:1 cell ratio) with gold nanospheres that were coated by a layer of PEG molecules conjugated to anti-D7-FIB antibody, which has high affinity to a 112 kDa antigen found on the HNDF plasma membrane [48]. After washing off the unbound gold nanospheres from the culture medium, the cells were irradiated by a series of 20 ultrashort



**Fig. 1. Myotube formation in HSMC culture and in HSMC-HNDF co-culture.** (a) Phase contrast image of myotubes formed by differentiated HSMC cells only. (b) Myotubes formed in an HSMC and HNDF (green GFP fluorescence) co-culture (1:1 cell ratio). White arrows point to the myotubes, which appear thinner in the presence of the HNDF cells.

pulses (45 fs pulse duration,  $7 \times 10^{10}$  W/cm<sup>2</sup>, 1 kHz repetition rate) that were tuned to match the plasmonic resonance wavelength of the gold nanospheres (550 nm).

An exemplary fusion process between an HSMC cell and two HNDF cells was captured in a time-lapse image series ([Visualization 1](#)), with three selected frames shown in Fig. 2. The movie reveals the relatively fast incorporation of one of the HNDF cells (HNDF 1) into the larger HSMC cell (cell boundaries are marked by a dashed line), staining the entire cytoplasm of the fused heterokaryon by the green fluorescence. The integration of the second HNDF cell (HNDF 2) into the HSMC cell was more gradual, and its nucleus remains visible within the resulting HNDF-HSMC heterokaryon cell throughout the entire movie. The three nuclei of the resulting fused cell are marked by three arrows on the right panel, 3 hours after irradiation.

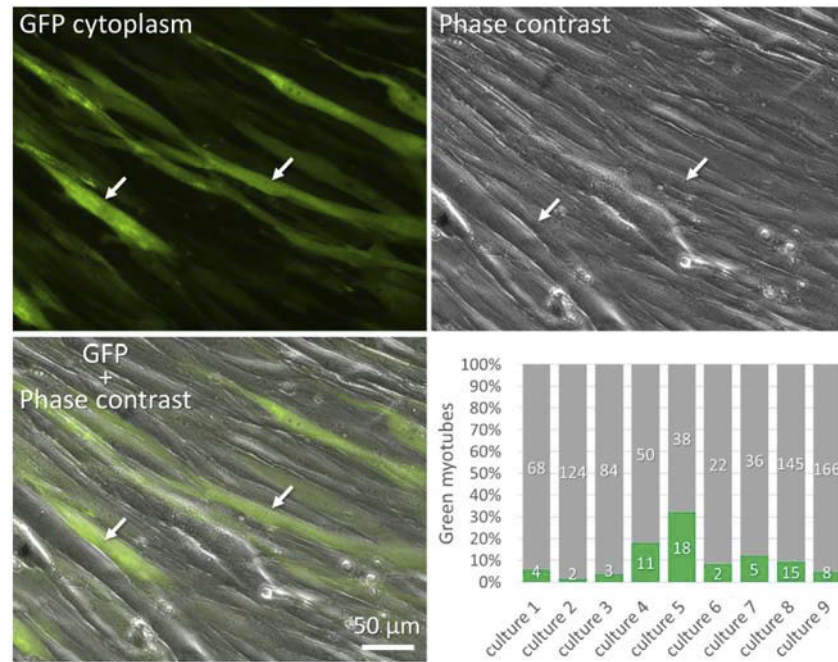


**Fig. 2. Plasmonic fusion between an HSMC cell and two HNDF cells.** Immediately after irradiation, the HSMC cell (boundaries marked by a dashed curve) becomes fluorescent after incorporating the two fluorescently labelled HNDF cells. Three hours after irradiation the resulting heterokaryon cell (dashed curve in the rightmost panel) showed a uniform green cytoplasm and contained three nuclei (arrows), indicating the completion of the successful fusion. The entire fusion process is shown in [Visualization 1](#).

In order to maintain the viability of the heterokaryon cells and induce effective differentiation into myotubes, the irradiated cultures were covered by Matrigel and incubated for 4 days in a serum-depleted medium. After 5 additional days of recovery in a standard growth medium (total of 9 days after laser irradiation), the long myotubes were already covering the entire culture (Fig. 3). Among all myotubes identified (based on cell volume and length, visible striations and number of nuclei [46]) in all nine irradiated cultures, an average of 8% of the cells were stained by the green fluorescence (Fig. 3, bottom-right bar chart), indicating their origin as the fusion product between the HNDF and the HSMC cells. Note that the ratio of the heterokaryon



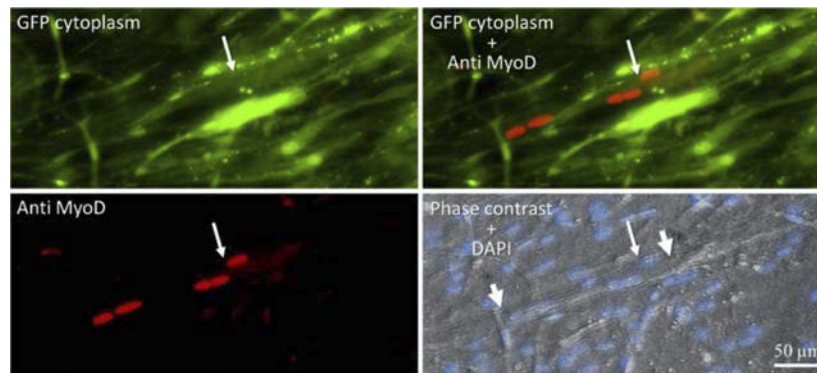
myotubes varied substantially among the different cultures, ranging from only 1.6% of the cells in culture #2 and up to 32% efficiency in culture #5.



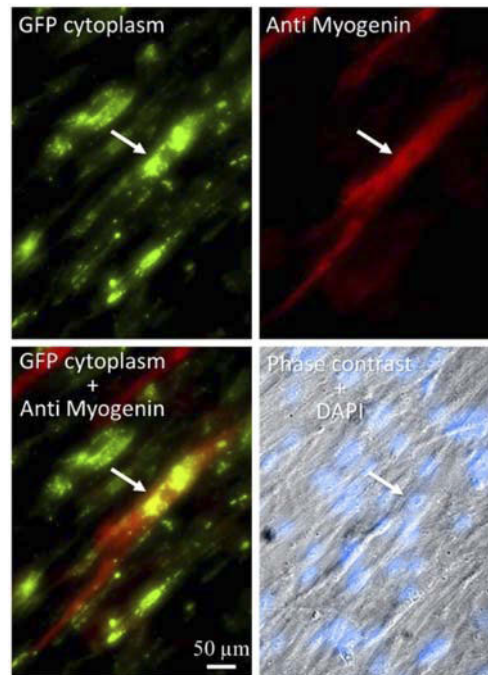
**Fig. 3. Efficiency of generating the heterokaryon myotubes.** Co-cultured HSMM cells and nanoparticle-targeted HNDF cells (Green, GFP labelled) nine days after laser irradiation have formed several elongated, multinucleated myotubes throughout the cell culture. White arrows point to the green labelled myotubes that originated from the fusion between the HSMM and HNDF cells. The bar chart shows the percentage of green myotubes (green columns) from the total number of myotubes in each culture. The number on the bars denotes the actual number of myotubes.

In order to verify that the resulting HNDF-HSMM heterokaryons possess the qualities of skeletal muscle cells, i.e. express proteins that are unique to muscle cells and take part in the sarcomeric structure of the myotube, the cells were fixated and their membranes were perforated prior to incubation with the staining antibodies. Due to the compromised plasma membranes, a large part of the cytoplasmic green fluorescence within the heterokaryon cells had leaked out, decreasing the brightness and uniformity of the green staining (see Fig. S1); yet the remaining green fluorescence was still sufficient for identifying these cells as HNDF-HSMM heterokaryons. Exposing the cell culture to antibodies raised against the principal transcription factor of muscle cells MyoD [40], resulted in cells showing (Fig. 4) both green (GFP) cytoplasm and red (Cy5) anti-MyoD secondary antibody labelling, indicating the expression of the MyoD protein in the heterokaryon cells. Additional examples of myotubes stained for both cytoplasm and MyoD are shown in Fig. S2. Note that the anti-MyoD staining could be found either inside the nucleus or within the entire cytoplasm of the myotube, depending on the cell cycle and its differentiation stage [49].

The presence of the Myogenin transcription factor [41] was tested for verifying the cells' entry into the differentiation pathway toward the formation of myotubes. By exposing the fixated and perforated cells to anti-Myogenin and to a secondary antibody conjugated to the fluorophore Cy5, the large myotubes have clearly shown (Fig. 5) the Cy5 staining within the green cells. Additional examples of myotubes stained for both GFP and Cy5 are shown in Fig. S3.



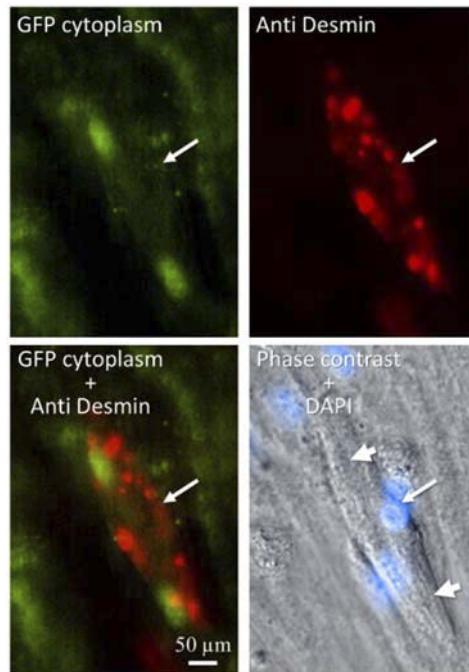
**Fig. 4. MyoD expression and striations in the heterokaryon myotubes.** Arrows mark a green (GFP) myotube of HNDF origin that is also stained for MyoD (Cy5, red). Cell nuclei in the phase contrast image are stained blue (DAPI). Visible striations are marked by short arrows in the phase contrast image.



**Fig. 5. Expression of Myogenin in the generated myotubes.** Arrows mark a green (GFP, HNDF origin) fluorescence myotube that is also stained for Myogenin (Cy5, red). The two markers within the myotube prove its dual fibroblastic and myoblastic origin. Cell nuclei in the phase contrast image are stained blue (DAPI).

The heterokaryon myotubes were further examined for their developmental stage by testing the expression of the intermediate filament protein Desmin, which is known to be basally expressed at low levels in satellite cells [50] and in myoblasts. [51] In mature myotubes it serves to connect between the z-disk of the sarcomere to the sarcolemma and the extra-cellular matrix. [52] The presence of the red (Cy5 fluorescence) anti Desmin secondary antibody within the green myotubes is shown in Fig. 6 and in Figure S4, indicating stable sarcomeric structures within

the myotubes, [53] structures which are also perceived as visible striations in the phase contrast images of the cells (short arrows in the bottom-right panel).

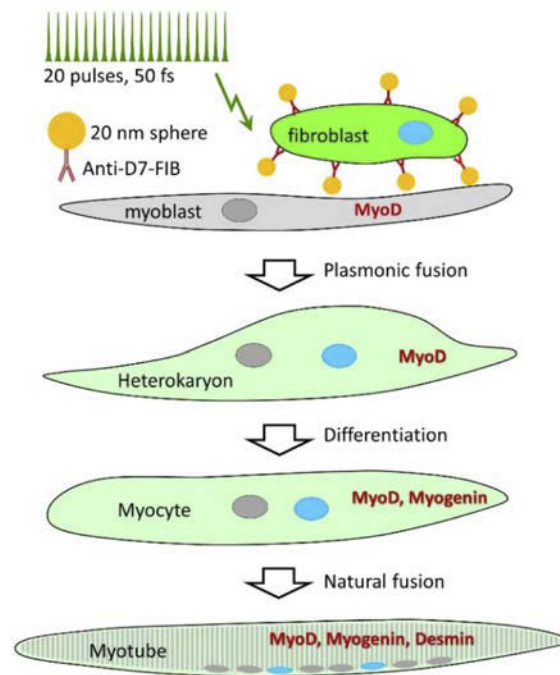


**Fig. 6. Myotubes of HNDF origin show Desmin expression and striations.** A large HNDF-origin myotube (arrows) with expression of Desmin (Cy5, red), indicating the formation of sarcomeres held by the intermediate filament Desmin. Cell nuclei in the phase contrast image are stained blue (DAPI). Short arrows point to visible striation in the phase contrast image.

#### 4. Discussion

Plasmonic fusion between human skeletal myoblasts and dermal fibroblasts yields the generation of viable myotubes that could develop and form a new muscle tissue. An illustrative model of the entire process is shown in Fig. 7 that summarizes the four key steps demonstrated in this work, including myoblast-fibroblast co-culturing, nanoparticle delivery and irradiation by a series of femtosecond pulses, cell-cell fusion, the formation of heterokaryon myocytes, and the generation of functional, striated myotubes.

The laser-triggered fusion has generated numerous heterokaryon cells within each culture, cells in which the myoblastic genetic information dominated the fibroblastic one, suggesting reprogramming of the resulting fused cell into a myoblastic lineage. We have found that the fused heterokaryons were expressing the MyoD myogenic regulatory factor, and then differentiated (following serum deprivation) into myocytes that display Myogenin expression, demonstrating commitment to myogenesis. The fused cells were eventually developing into myotubes, as evident by the expression of Desmin and by the visible striations that are a characteristic of sarcomeric structural elements. [52] All of the GFP labelled myotubes within the irradiated cultures, i.e. all HNDF-HSMM fusion products, have shown clear staining of MyoD, Myogenin and Desmin, and have remained viable for at least 9 days after irradiation by the laser pulses. The ratio between the green heterokaryon myotubes and the total myotube population (which also includes unfused cells) ranged between 1.6–32% in the different experiments, variations that most likely depend on



**Fig. 7.** An illustrative model for HNDF-HSMM cell fusion for a heterokaryon myotube generation using plasmonic cell fusion.

the specific cell physiology, amount of neighboring HNDF and HSMM cells within the culture, nanoparticle attachment efficiency, and occasional variations in laser power.

The method for producing myotubes from HNDF-HSMM plasmonic fusion may benefit various advanced therapies that involve cell transplantation into the damaged muscles. Studies on the regeneration of damaged skeletal muscle in dystrophic *mdx* and *dy/dy* mice, [21,54–56] followed by studies in large animals [56,57] and human clinical trials, [58,59] have been mostly based on allogeneic or autologous transplantation of either satellite cells or satellite cells that were *in vitro* expanded into myoblasts. For localized muscle damage, multiple high-density injections in combination with immunosuppression medication have shown effective restoration of dystrophin expression in patients. [59,60] Furthermore, co-injection of myogenic progenitor cells and bone marrow mesenchymal stem cells have shown improved functionality of the injected limb. [61] In cases of extensive muscle damage, however, the various transplantation methods often become less effective in generating new muscle tissue. Our method for *ex-vivo* generation of a myoblast-fibroblasts co-culture where the fibroblast cells account for at least 50% of the cell mass and provide important biological support for the myoblasts, could be used for cell transplantation in the context of these methods, or even as a standalone technique for regenerating the damaged muscle tissue.

Other approaches for treating extensive muscle loss include the use of cell sheet-derived engineered muscle grafts, [62,63] acellular scaffolds in human patients, [64,65] minced autologous or allogeneic muscle grafts combined with a hydrogel support, [66,67] and muscle grafts engineered with multiple cell types and scaffolds. [68] The myotubes generated by plasmonic fusion may be used for therapy in a similar manner, for example by deriving fibroblast cells and myoblasts from the patient, fusing them together to form viable myotubes, stabilizing the resulting cell culture in a hydrogel, and transplanting the three-dimensional culture into the damaged muscle. Such approach may reduce the size of necessary muscle biopsies, and may include other



cell types or various hydrogel configurations [66] for supporting normal vascularization and neuro-muscular junction development, which are imperative for normal muscle function.

Several challenges still remain before this technique could be tested for clinical applications. First, the overall efficiency of generating healthy myoblasts would need to be improved for achieving effective treatment. In numerous control experiments we have verified that the expressions of MyoD, Myogenin and Desmin in the HSMM cells were unaffected by the presence of the gold nanoparticles (Fig. S5) and by the laser irradiation levels. The generation of viable heterokaryon myotubes may also benefit from optimizing the fusion process, for example by utilizing bi-specific nanoparticles [34] that could promote physical attachment between the HSMM and the HNDF cells, creating pre-conditioned cell pairs prior to laser irradiation. However, the HSMM cells were found to be extremely sensitive in our experiments, forcing us to design nanoparticles that target only the HNDF cells. Additional experiments may be required in order to further optimize particle concentration and incubation times, aiming to explore different cell ratios and densities, and to calibrate the various irradiation parameters to the new cell cultures. Second, after fusion, the resulting heterokaryon cells may benefit substantially from an environment that better supports the formation of functional, contracting myotubes. In addition to the Matrigel used in our experiments, which have been shown useful for guiding muscle regeneration, [45,46,69] numerous studies [70,71] have shown that on soft patterned substrates the myotubes often exhibit better alignment with distinct striated pattern that undergo spontaneous twitching. A micropatterned substrate may thus improve myotube development, particularly in the presence of the lining HNDF cells [24,72] that were found to improve myotube alignment in our experiments (see Fig. 1) (see also Ref. [46]). Finally, further experiments would be needed to test the feasibility of plasmonic fusion for muscle tissue regeneration, including the introduction of controlled mechanical damage to the myotube culture using a scratch test [73] or the application of an excessive stretch, [74] followed by close observation of the culture recovery following plasmonic fusion. Other means for introducing controlled damage to the culture may include simulating the oxidative stress that occurs following extensive exercise, [74] or adding various drugs (e.g. cholesterol lowering statins [75]) to the culture medium. Such experiments may prove the ability of the fused HNDF-HSMM cells to overcome cell damage and regenerate the myotube culture with the aid of the dermal fibroblasts.

In conclusion, we have utilized plasmonic cell fusion for generating viable myotubes with a mixed myoblastic and fibroblastic source. Laser pulse irradiation of the HSMM-HNDF co-culture yielded numerous heterokaryon cells that express MyoD, Myogenin and Desmin, indicating that the cells may be functioning as viable myotubes. The resulting process of fibroblast reprogramming into functional myotubes may be used to assist various therapeutic approaches for regenerating muscle tissue in patients suffering from muscle loss or muscle dystrophy.

**Disclosures.** The authors declare no conflict of interests.

**Data availability.** No data were generated or analyzed in the presented research.

**Supplemental document.** See [Supplement 1](#) for supporting content.

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